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ORIGINAL ARTICLE

The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling

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The mechanisms underlying the initiation/onset of, and the recovery from, depression are still largely unknown; views that neurogenesis in the hippocampus may be important for the pathogenesis and amelioration of depressive symptoms have gained currency over the years although the original evidence has been challenged. In this study, an unpredictable chronic mild stress protocol was used to induce a depressive-like phenotype in rats. In the last 2 weeks of stress exposure, animals were treated with the antidepressants fluoxetine, imipramine, CP 156,526 or SSR 1494515, alone or combined with methylazoxymethanol, a cytostatic agent used to arrest neurogenesis. We found that antidepressants retain their therapeutic efficacy in reducing both measured indices of depression-like behavior (learned helplessness and anhedonia), even when neurogenesis is blocked. Instead, our experiments suggest re-establishment of neuronal plasticity (dendritic remodeling and synaptic contacts) in the hippocampus and prefrontal cortex, rather than neurogenesis, as the basis for the restoration of behavioral homeostasis by antidepressants.
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Introduction

Neuronal plasticity and neurogenesis in the hippocampus are two, not necessarily mutually exclusive, mechanisms proposed to underlie the behavioral improvement seen in response to antidepressant treatments.[1–3] Neurogenesis occurs in many mammals,[4,5] including humans;[6] even thought this process is generally viewed as a crucial promoter of neuronal plasticity, some studies have recently disputed the relevance of neurogenesis for certain behavioral adaptations.[7] Studies showing that hippocampal neurogenesis is reduced in animal models of depression, and stimulated by a range of antidepressants,[8–11] led to the proposal that neurogenesis may have a role in the pathogenesis of depression. The importance of altered neurogenesis in the precipitation of depression-like behavior has, however, been challenged recently.[12,13] Questions have also been raised[14–16] regarding an earlier proposal by Santarelli *et al.*,[17] that hippocampal neurogenesis is essential for the manifestation of behavioral improvement after the administration of antidepressants. In contrast, stress, an important trigger of depression, is known to reduce neurogenesis[18] and impair synaptic plasticity and dendritic arborization in the hippocampus[19] as well as in 'executive centers' such as the prefrontal cortex (PFC).[20–22] Antidepressants attenuate hippocampal volume loss in depressed patients[23] and can reverse some of the deleterious effects of stress on synaptic/dendritic structure.[24]

In this study, we therefore analyzed the neurotrophic hypothesis of depression in its wider context, focusing on hippocampal neurogenesis and neuronal plasticity in the hippocampus and PFC. An unpredictable chronic mild stress (CMS) paradigm was used for 6 weeks to induce core symptoms of depressive-like behavior in rats.[25] Anhedonia, learned helplessness and anxiety-like behaviors were assessed using standardized assays (sucrose preference test, forced swimming test (FST) and novelty suppressed feeding (NSF) test, respectively) at the end of the experimental period. During the last 2 weeks of CMS, standard antidepressants of two different classes (imipramine and fluoxetine) and two putative antidepressants (CP 156,526, a type 1 corticotropin-releasing hormone receptor antagonist, and SSR 1494515, a type 1b arginine vasopressin receptor antagonist) were administered daily. To evaluate the requirement of hippocampal neurogenesis for the

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behavioral effects of antidepressants, the cytostatic agent methylazoxymethanol (MAM) was co-administered with these drugs in different subgroups of animals. All animals received a single injection of bromodeoxyuridine (BrdU) 1 day before killing and samples of the hippocampus and PFC were prepared for immunocytochemical analysis of proliferation (BrdU and Ki-67) and differentiation markers (doublecortin (DCX), neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP)), stereological estimations of total number of neurons and volumes, Golgi impregnation to assess dendritic arborization and spine shape and number by three-dimensional morphometry, and measurement of mRNA levels of the synaptic remodeling/plasticity proteins neural cell adhesion molecule 1 (NCAM1) and synapsin 1 (SYN1).

Materials and methods

Animals

Male Wistar rats (Charles-River Laboratories, Barcelona, Spain), weighing 300–400 g and aged 3 months were used in this study. Animals were housed (three per cage) under standard laboratory conditions (12 h light/12 h dark cycle, at 22 °C, relative humidity of 55%; free access to food and water). Animals were assigned to one of two main treatment groups (control and CMS—see below). All procedures were carried out in accordance with European Union Directive 86/609/EEC and NIH guidelines on animal care and experimentation.

Drugs

The drugs used were fluoxetine (10 mg kg^{-1}; Kemprotec, Middlesborough, UK), imipramine (10 mg kg^{-1}; Sigma-Aldrich, St Louis, MO, USA), CP 156,526 (20 mg kg^{-1}; Pfizer, New York, NY, USA) and SSR 149415 (30 mg kg^{-1}; Sanofi-Synthelabo, Montpellier, France) and MAM (7 mg kg^{-1}; National Cancer Institute, Midwest Research Institute, Kansas City, MO, USA). Fluoxetine, imipramine, CP 156,526 and SSR 149415 were administered intraperitoneally (i.p.; 1 ml kg^{-1}); MAM was administered subcutaneously (0.45 ml kg^{-1}). Compounds were dissolved in 5% DMSO in 0.9% saline. All animals received an injection of BrdU (100 mg kg^{-1} i.p.) 24 h before killing. As MAM is a powerful cytostatic drug, we were concerned about the potential confounding effects of blocking mitosis in organs other than the brain, such as the epithelium of the intestine.[26] Pilot studies showed that MAM at a dose of 7 mg kg^{-1} per day reduces neurogenesis (BrdU and Ki-67 immunostaining) by approximately 60% in the dentate gyrus of all (including CMS) experimental groups. As an index of general health,[27,28] we assessed locomotor activity (ambulation in an open field, swimming speed in a water maze) and fur quality; MAM-treated and control rats scored equally on these end points.

Chronic mild stress

A slightly modified version of a CMS protocol[25] was used. It consisted of chronic exposure to unpredictable mild stressors (confinement to a restricted space for 1 h, placement in a tilted cage (30°) for 3 h, housing on damp bedding for 8 h, overnight illumination, 18 h food deprivation followed by exposure to inaccessible food for 1 h, water deprivation for 18 h followed by exposure to an empty bottle for 1 h, and reversed light/dark cycle for 48 h every 7 days) over 6 weeks. During the last 2 weeks of CMS, animals were given daily injections of either saline (CMS + saline), saline + MAM, or one of the four antidepressants with or without MAM; each of these subgroups consisted of 24 animals; in addition, saline- or MAM-treated control animals that were not exposed to CMS were also used ($n = 48$).

Sucrose preference test

Anhedonia was assessed weekly during exposure to CMS using the sucrose preference test. Briefly, animals were allowed to habituate to the sucrose solution 1 week before the CMS protocol to establish baseline preference levels. To test sucrose preference, animals that were food- and water-deprived for 18 h were presented with two preweighed bottles containing 1% sucrose solution or tap water for a period of 1 h. Sucrose preference was calculated according to the formula: sucrose preference = (sucrose intake/(sucrose intake + water intake)) × 100, as previously described.[29] Anhedonia was defined as a reduction in sucrose preference relative to baseline levels.

Forced swimming test

Learned helplessness was evaluated in the FST on the last day of exposure to CMS. Twenty-four hours after a pretest session (10 min), the FST was conducted by placing rats in cylinders filled with water (25 °C; depth 30 cm) for a period of 5 min. Test sessions were assessed using a camera connected to a video tracking system (Viewpoint); the system automatically calculated immobility time and latency to immobility. Learned helplessness behavior was defined as an increase in time of immobility and a decrease in latency to immobility.

Novelty suppressed feeding

To characterize anxiety-like behavior, NSF was assessed on the last day of the CMS paradigm. This test was conducted in an independent group of animals prepared as before (controls ± MAM; CMS ± MAM; CMS ± fluoxetine or imipramine or CP 156,526 or SSR 149415; CMS ± fluoxetine or imipramine or CP 156,526 or SSR 149415 ± MAM; $n = 12$ per group). Based on previous studies,[30] animals were deprived of food for 23 h before being placed in a novel environment for 10 min (an open-field arena; MedAssociates Inc.); a single food pellet was placed in the center of the arena. Upon reaching the pellet, animals were returned to their home cages and presented with preweighed food over a period of

5 min. Latency to feeding in the open field was used as an index of anxiety-like behavior; the amount of food consumed in the home cage provided a measure of appetitive drive.

Immunostaining procedures

Animals were rapidly decapitated and brains fixed in 4% paraformaldehyde. Serial coronal sections, extending over the entire length of the telencephalon, were cut and stained for BrdU (1:50; Dako, Glostrup, Denmark) or Ki-67 (1:200; Novocastra, Newcastle-upon-Tyne, UK). BrdU is incorporated into DNA only during the S-phase of the mitotic process; Ki-67 is a nuclear protein expressed in all phases of the cell cycle except for the resting phase.[31] Sections were then double stained for DCX (for neuroblasts; 1:500; Abcam, Cambridge, UK), NeuN (for neurons; 1:100; Chemicon, Temecula, CA, USA) or GFAP (for glia; 1:200; Dako). A universal detection system (BioGenex, San Ramon, CA, USA) and diaminobenzidine (0.025% and 0.5% H_2O_2 in Tris–HCl 0.05 M, pH 7.2) were used to visualize immunostained cells after counterstaining with hematoxylin. Proliferation densities were estimated in the subgranular zone (SGZ; defined as a two-cell layer-thick zone on the inner side of the granule cell layer of the dentate gyrus), using an Olympus BX51 optical microscope and Newcast software (Visiopharm). The density of BrdU, Ki-67 labeled cells in the SGZ was estimated in every eighth section, as a ratio between the total number of immunostained cells and the volume of the SGZ. For each animal, 50–100 BrdU and/or Ki-67-positive cells within the SGZ of the dentate gyrus were analyzed after double staining with neuronal (DCX or NeuN) or glial (GFAP) markers, using a confocal microscope (Olympus FV1000).

Structural analysis

Total number of neurons in the hippocampal formation and in the medial PFC (mPFC) were estimated using the optical fractionator (see Cerqueira *et al*.[22] for details) in the right hemisphere of six animals from each experimental group. Brains were postfixed in 4% paraformaldehyde for 48 h and embedded in glycolmethacrylate. Volumes were determined by the Cavalieri's principle using the StereoInvestigator software (Microbrightfield). For the three-dimensional morphometric analysis, six animals from each treatment group were transcardially perfused with 0.9% saline and processed.[32] Briefly, brains were immersed in Golgi-Cox solution[33] for 21 days; transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated and xylene cleared before coverslipping. Dendritic arborization and spine numbers and shape were analyzed in the dentate gyrus, CA3 region of the hippocampus and layer II/III of the prelimbic area of the mPFC. For each selected neuron, all branches of the dendritic tree were reconstructed at $\times 600$ (oil) magnification using a motorized microscope (Axioplan 2; Carl Zeiss) and Neurolucida software (Microbrightfield). A three-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). For each animal, 40 neurons were studied and measurements from individual neurons from each animal were averaged. Several aspects of dendritic morphology were examined. Total dendritic length and the number of primary dendrites and dendritic branching points were compared across experimental groups. Dendritic spine density (number of spines/dendritic length) was determined in branches that were either parallel or at acute angles to the coronal surface of the section. In dentate granule cells, proximal and distal branches were analyzed for each neuron; basal branches and proximal and distal apical branches in pyramidal neurons in the CA3 region and prelimbic area of the mPFC were analyzed. Three-dimensional Sholl analysis was used to evaluate the arrangement of the dendritic material; for this, the number of dendritic intersections with concentric spheres positioned at radial intervals of 20 μm was determined. To assess changes in spine morphology, spines in the selected segments were classified into mushroom-shaped, thin, wide and ramified spines[34] and the proportion of spines in each category was calculated for each neuron.

qPCR measurements

Levels of *Ncam1* and *Syn1* mRNA expression were determined by quantitative PCR (qPCR) in hippocampi and PFC derived from four animals of each treatment group. Total RNA (2 μg) were reverse transcribed using oligo-dT primers of the Superscript First-strand Synthesis system for reverse transcription–PCR (Invitrogen, Carlsbad, CA, USA). Hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used as internal standard for normalization. Oligonucleotide primers for *Ncam1* (sense AAAGGA TGGGGAACCCATAG, antisense TAGGTGATTTTG GGCTTTGC), *Syn1* (sense CACCGACTGGGCAAAAT ACT, antisense TCCGAACTTCCATGTCC) and *Hprt* (sense GCAGACTTTGCTTTCCTTGG, antisense TCCACTTTCGCTGATGACAC) were designed using the Primer3 software on the basis of the GenBank sequences NM_031521, NM_019133 and NM_012583, respectively. Real-time PCR reactions were performed on a LightCycler (Roche Diagnostics, Basel, Switzerland), using QuantiTect SYBR Green RT-PCR (Qiagen, Hilden, Germany).

Statistical analysis

After confirmation of homogeneity, appropriate statistical tests were applied to the data. Repeated measures ANOVA were used to analyze sucrose preference test results, body mass and physical state of the fur. Two-factor ANOVA was used to evaluate

other behavioral data as well as cell densities and immunostaining results. Differences between groups were then determined by Tukey's honestly significant difference test (Tukey HSD) post hoc analysis. Statistical significance was accepted for $P<0.05$. Results are expressed as mean ± s.e.m.

Results

Behavioral results

Anhedonia was assessed weekly in the sucrose consumption test. Sucrose preference was reduced in CMS animals ($F_{4,176}=279.25$, $P<0.001$). This anhedonic phenotype was reversed by imipramine, CP 156,526 and SSR 149415 after the first, and by fluoxetine after the second week of treatment ($P<0.001$ in all cases; Figure 1a). Repeated measures ANOVA failed to reveal an interaction between antidepressant treatment and co-administration of MAM ($F_{8,\ 220}=0.95$, $P=0.469$). The FST revealed a significant increase in immobility time in stressed animals ($F_{1,\ 44}=18.56$, $P<0.001$). Chronic administration of fluoxetine ($P=0.006$), imipramine ($P<0.001$), CP 156,526 ($P<0.001$) and SSR 149415 ($P<0.001$) reversed this sign of depressive-like behavior even when MAM was administered (Figure 1b). Exposure to CMS significantly increased the latency to feed in the NSF paradigm ($F_{1,\ 44}=5.02$, $P=0.03$), an effect that was reversed by fluoxetine, imipramine and CP 156,526 ($P<0.001$ in all cases), confirming the anxiolytic properties of these drugs (Figure 1c). The co-administration of MAM increased this measure in all experimental groups ($P<0.001$).

Cell proliferation and differentiation

The density of cells labeled for the endogenous marker of proliferation, Ki-67, was significantly reduced in animals exposed to CMS ($F_{1,\ 20}=29.77$, $P<0.001$; Figure 1d). Treatment with either fluoxetine, imipramine, CP 156,526 or SSR 149415 restored this parameter to control levels ($P<0.001$ in all cases). The density of Ki-67-positive cells was significantly reduced in animals receiving concomitant injections of MAM ($P<0.001$). The percentage of Ki-67-immunopositive cells that colabeled with antibodies against DCX (a marker of early neuroblasts) in the SGZ of the dentate gyrus was significantly lower in CMS-treated rats ($F_{1,\ 20}=13.72$, $P=0.001$) but reversed by fluoxetine ($P=0.001$), imipramine ($P=0.008$), CP 156,526 ($P=0.001$) and SSR 149415 ($P=0.005$; Figure 1e). The percentage of cells showing colocalization of Ki-67 and DCX was reduced in all groups receiving MAM ($P<0.001$).

The density of BrdU-positive cells in the SGZ was significantly decreased in animals exposed to CMS ($F_{1,\ 20}=29.6$, $P<0.001$), an effect reversed after chronic administration of fluoxetine, imipramine, CP 156,526 or SSR 149415 ($P<0.001$ in all cases; Supplementary Figure S1a). Administration of MAM significantly reduced the density of BrdU positive

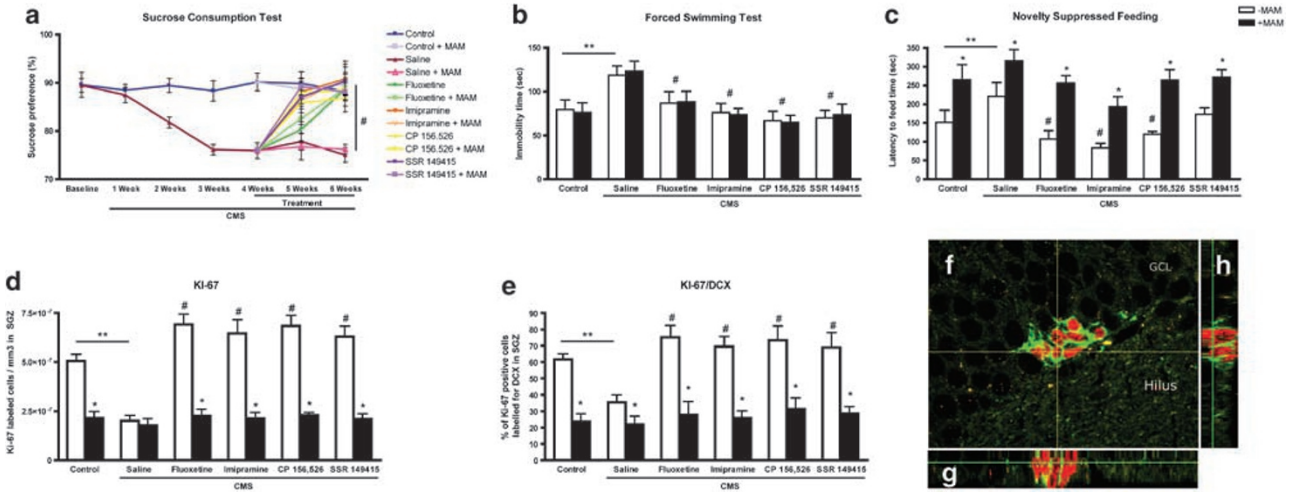

Figure 1 Amelioration of behavioral symptoms of depression by antidepressant drugs does not depend on neurogenesis. (**a**) Anhedonia was assessed weekly in the sucrose consumption test ($n=12$ per group; $^{*}P<0.001$). (**b**) Learned helplessness was evaluated in the forced swimming test (FST) at the end of the chronic mild stress (CMS) protocol ($n=12$ per group; $^{**}P<0.001$; $^{\#}P<0.006$). (**c**) Anxiety-like behavior in the novelty suppressed feeding paradigm at the end of the CMS protocol ($n=12$ per group; $^{*}P<0.001$; $^{**}P\leqslant 0.001$; $^{\#}P=0.03$). (**d**) The density of recently-mitotic cells labeled for the endogenous marker of proliferation Ki-67 in the subgranular zone (SGZ) of the dentate gyrus ($n=6$ per group; $^{*}P<0.001$; $^{**}P<0.001$; $^{\#}P\leqslant 0.001$). (**e**) Percentage of Ki-67-immunopositive cells that co-labeled with antibodies against doublecortin (DCX, a marker of early neuroblasts) in the SGZ ($n=6$ per group; $^{*}P<0.001$; $^{**}P=0.001$; $^{\#}P<0,008$). (**f**) Image of a niche of newly formed neurons in the SGZ, obtained by confocal microscopy. Red: Ki-67-positive cells; green: DCX-positive cells. Confocal stacks (image shown in **f**) resliced in the *x*-*z* plane (**g**) and in the *y*-*z* plan (**h**). Data represented as mean ± s.e.m. Asterisk represents the effect of methylazoxymethanol (MAM) in every experimental group. Double asterisk represents the comparison between control and CMS groups. Cardinal represents the effect of antidepressant treatment.

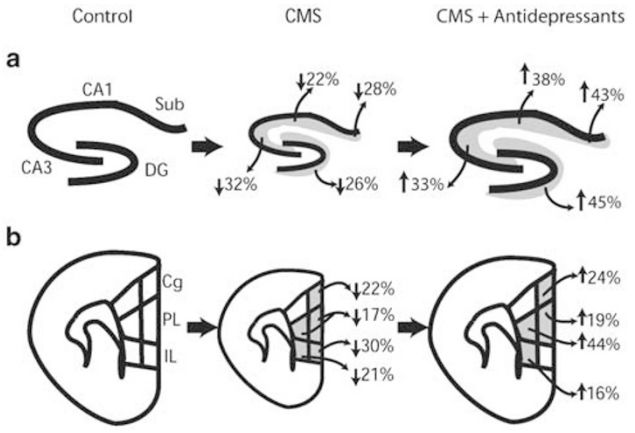

Figure 2 Schematic representation of structural changes in the hippocampus (**a**) and medial prefrontal cortex (**b**). Chronic mild stress (CMS) induced a volumetric reduction in the molecular layer of the dentate gyrus and strata radiatum of CA3, CA1 and subiculum; these atrophic changes were reversed by all antidepressants ($n=6$ per group; for all regions $P<0.05$). Exposure to stress induced volumetric reductions in the medial prefrontal cortex (mPFC), specifically in the superficial layers of the cingulum (Cg), prelimbic (PL) and infralimbic (IL) regions, as well as in deep layers of the PL and IL. All antidepressants efficiently promoted volumetric recovery in all these layers ($n=6$ per group; for all regions $P<0.05$) to a similar extent (indicated changes in percentage refer to those found in CMS + fluoxetine-treated animals). None of the experimental groups showed significant neuronal loss in either the hippocampus or mPFC (data not shown).

cells in control and antidepressant-treated animals ($P<0.001$), thus confirming antiproliferative effect of this cytostatic drug. The percentage of BrdU-positive cells that double-labeled with anti-NeuN in the SGZ was decreased by exposure to CMS ($F_{1,\ 20}=8.51$, $P=0.008$; Supplementary Figure S1b). The chronic administration of fluoxetine ($P=0.006$), imipramine ($P=0.01$), CP 156,526 ($P=0.001$) and SSR 149415 ($P=0.005$) reversed this change. Administration of MAM significantly reduced the percentage of BrdU- and NeuN-positive cells in all experimental groups ($P<0.001$). The percentage of BrdU-positive cells labeled with GFAP in the SGZ was not significantly altered after exposure to CMS, or after administration of antidepressant drugs or MAM, alone or in combination (Supplementary Figure S1c).

Structural analysis

As shown in Figure 2a, CMS induced a volumetric reduction in the molecular layer of the dentate gyrus (26%) and strata radiatum of CA3 (32%), CA1 (22%) and subiculum (Sub; 28%); these atrophic changes were reversed by all antidepressants (for all regions $P<0.05$). CMS also induced volumetric reductions in the mPFC (Figure 2b), specifically in the superficial layers of the cingulum (Cg), prelimbic (PL) and infralimbic (IL) regions, as well as in deep layers of the PL and IL. Again, all antidepressants tested efficiently promoted volumetric recovery in all these layers (for all regions $P<0.05$) to a similar extent. None of the experimental groups showed significant neuronal loss in either the hippocampus or mPFC (data not shown).

The three-dimensional morphometric analysis of Golgi-impregnated neurons revealed that CMS induces significant atrophy of granule cell dendrites ($F_{1,\ 20}=32.06$, $P<0.001$) and CA3 pyramidal neurons ($F_{1,\ 20}=46.35$, $P<0.001$; Figure 3d and e), as measured by total dendritic lengths. Administration of either fluoxetine, imipramine, CP 156,526 or SSR 149415 reversed this effect in both regions ($P<0.005$ in all cases). Significant dendritic atrophy was also observed in pyramidal neurons in the PFC after exposure of animals to CMS ($F_{1,\ 20}=130.09$, $P<0.001$; Figure 3f); these changes were attenuated by concomitant treatment with imipramine, CP 156,526 and SSR 149415 ($P<0.001$ in all cases) but not by fluoxetine. The dendritic atrophy observed in CA3 and PFC pyramidal neurons, and its reversal by antidepressant treatment, was confined to apical dendrites; basal dendritic lengths were not significantly altered by any of the treatments (Supplementary Figure S2). None of the treatments resulted in changes in the density of spines in dendrites of the dentate granule neurons (Figure 3g). In contrast, CMS was associated with significant loss of spines in the proximal, but not distal, dendrites of CA3 pyramidal neurons ($F_{1,\ 20}=25.83$, $P<0.001$; Figure 3h); this effect was attenuated by fluoxetine ($P=0.001$), imipramine ($P<0.001$), CP 156,526 ($P=0.011$) and SSR 149415 ($P<0.001$) treatment. Decreased spine densities in the proximal ($F_{1,\ 20}=84.86$, $P<0.001$) and distal ($F_{1,\ 20}=134.89$, $P<0.001$) dendrites of PFC pyramidal neurons were observed in CMS-treated rats (Figure 3i). All antidepressant drugs tested reversed these changes ($P<0.001$, for proximal and distal dendrites in all cases). The CMS protocol did not cause spine density changes in the basal dendrites of hippocampal or PFC pyramidal neurons (data not shown). The number of dendritic branch points was significantly reduced in the dentate gyrus ($F_{1,\ 20}=11.503$, $P<0.001$) and PFC ($F_{1,\ 20}=45.629$, $P<0.001$) of animals exposed to CMS (Supplementary Figure S3a and c). In the dentate gyrus, this effect was reversed by fluoxetine ($P<0.001$), imipramine ($P<0.001$), CP 156,526 ($P=0.002$) and SSR 149415 ($P<0.001$); treatment with fluoxetine ($P=0.015$), imipramine ($P<0.001$), CP 156,526 ($P<0.001$) and SSR 149415 ($P<0.001$) attenuated the effects of stress in the PFC. Despite the lack of effect of CMS in the CA3 region (Supplementary Figure S3b), there was a significant increase in the number of dendritic branch points in stressed animal treated with CP 156,526 ($P=0.022$). The number of primary dendrites was not influenced by either stress exposure or treatment with antidepressants in any of the regions analyzed (Supplementary Figure S3d, e and f). Treatment with the antiproliferative agent MAM did not influence total dendritic length, spine

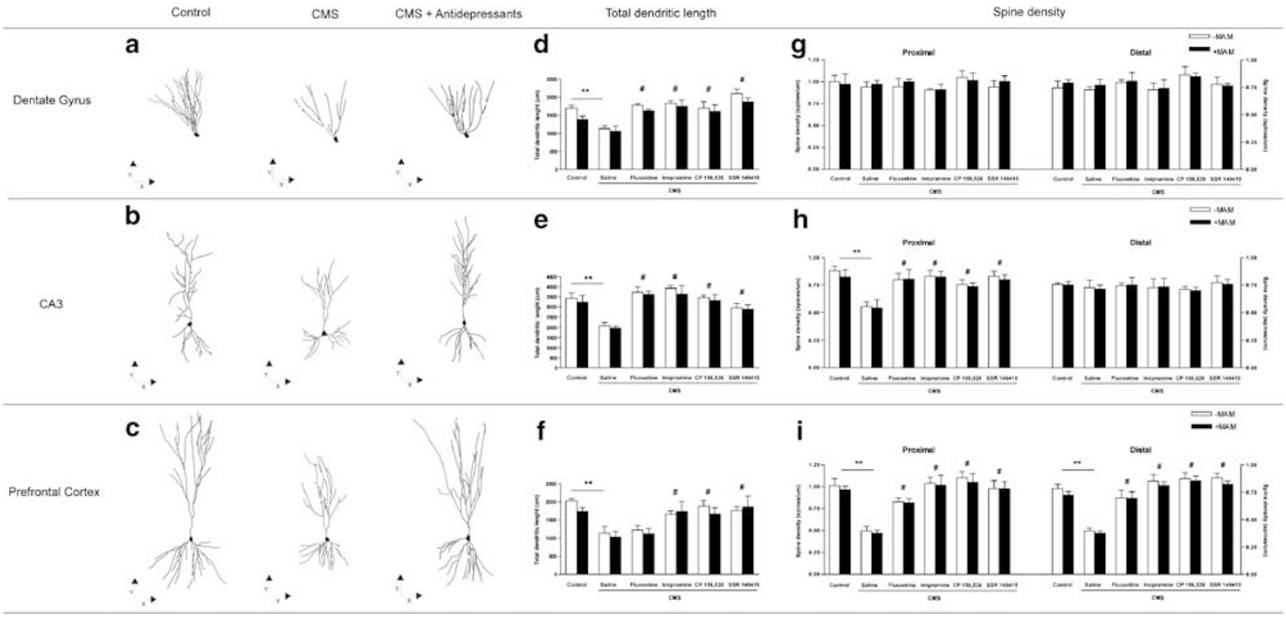

Figure 3 Three-dimensional morphometric analysis of Golgi-impregnated neurons using computer-assisted reconstructions of hippocampal and medial prefrontal cortex (PFC) neurons. Representative dentate granule (**a**), CA3 pyramidal (**b**) and layer II/III pyramidal neurons from the prelimbic area of the medial PFC (c) are shown. Cells are depicted in the *x*-*y* orthogonal plan. Total dendritic length of (**d**) dentate granule neurons in the subgranular zone (SGZ; $n=6$ per group; $^{**}P<0.001$; $^{\#}P\leqslant0.005$), (**e**) CA3 pyramidal neurons ($n=6$ per group; $^{**}P<0.001$; $^{\#}P\leqslant0.005$) and (**f**) pyramidal neurons in the PFC ($n=6$ per group; $^{**}P<0.001$; $^{\#}P\leqslant0.001$). Spine density in the proximal and distal dendrites of (**g**) dentate granule neurons in the SGZ, (**h**) CA3 pyramidal neurons ($n=6$ per group; $^{**}P<0.001$; $^{\#}P<0.011$) and (**i**) pyramidal neurons in the PFC ($n=6$ per group; $^{**}P<0.001$; $^{\#}P\leqslant0.001$). Data represented as mean ± s.e.m. Asterisk represents the effect of methylazoxymethanol (MAM) in every experimental group. Double asterisk represents the comparison between control and chronic mild stress (CMS) groups. Cardinal represents the effect of antidepressant treatment.

densities, number of dendritic branch points and primary dendrites (hippocampus and PFC) in any of the experimental groups. Sholl analysis of dendritic distribution revealed significantly fewer dendritic intersections in the dentate gyrus ($F_{1,20}=5.717$, $P=0.027$) and PFC ($F_{1,20}=53.891$, $P<0.001$) of stressed animals (Supplementary Figure S3g and i). In the dentate gyrus, the CMS effect was reversed by fluoxetine ($P=0.003$), imipramine ($P<0.001$), CP 156,526 ($P<0.001$) and SSR 149415 ($P<0.001$); similarly, fluoxetine ($P=0.001$), imipramine ($P<0.001$), CP 156,526 ($P<0.001$) and SSR 149415 ($P<0.001$) attenuated the effects of stress in the PFC. Although CMS did not induce any significant changes in the CA3 region (Supplementary Figure S3h), treatment with fluoxetine ($P=0.029$) and imipramine ($P=0.0028$) led to an increase in dendritic intersections. None of the experimental groups that additionally received the antiproliferative agent MAM showed changes in dendritic distribution in any of the areas evaluated (data not shown).

The morphological classification of dendritic spines revealed that the percentage of mushroom-type spines in the proximal dendrites of dentate granule neurons was significantly decreased in rats exposed to CMS ($F_{1,20}=8.06$, $P=0.01$); in contrast, CMS-treated animals displayed an increase in the percentage of thin-type spines ($F_{1,20}=258.35$, $P<0.001$; Supplementary Figure S4a). These changes were reversible after treatment with all of the antidepressants tested ($P<0.001$ in all cases). The percentage of mushroom-type spines in the distal dendrites of dentate granule neurons was also significantly decreased in CMS-exposed animals ($F_{1,20}=44.68$, $P<0{,}001$); both CP 156,526 ($P=0.004$) and SSR 149415 ($P=0{,}002$), but not fluoxetine or imipramine, reversed this effect (Supplementary Figure S4b). The percentage of thin-type spines was increased in CMS-treated rats ($F_{1,20}=273.52$, $P<0.001$) and restored to control levels by all antidepressants ($P<0.001$ in all cases). No changes were observed on the morphology of dendritic spines in CA3 pyramidal neurons (Supplementary Figure S4c and d). A significant increase in the percentage of thin-type spines in the proximal dendrites of pyramidal neurons of the mPFC was observed in animals exposed to CMS ($F_{1,20}=35.43$, $P<0.001$) (Supplementary Figure S4e); imipramine ($P=0.007$), CP 156,526 ($P=0.001$) and SSR 149415 ($P=0.005$), but not fluoxetine, reversed the changes induced by CMS. The percentage of mushroom-type spines was significantly decreased in the distal dendrites of pyramidal neurons of the PFC in animals exposed to CMS ($F_{1,20}=98.47$, $P<0{,}001$); these changes were normalized by all antidepressants ($P<0.006$ in all cases; Supplementary Figure S4f). In addition,

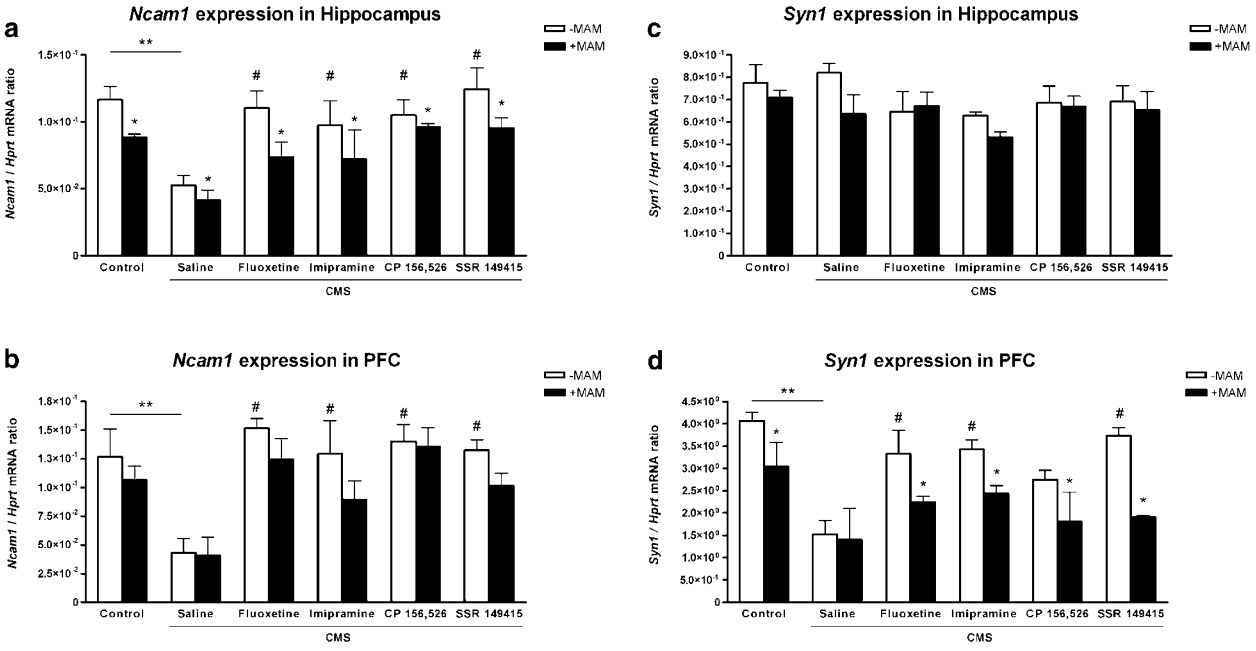

Figure 4 Antidepressant treatment induced changes in mRNA expression levels of the synaptic remodeling proteins neural cell adhesion molecule 1 (NCAM1) in (**a**) the hippocampus ($n=4$ per group; $^{**}P<0.001$; $^{\#}P<0.01$) and (**b**) the prefrontal cortex (PFC) ($n=4$ per group; $^{**}P<0.001$; $^{\#}P<0.01$) and synapsin 1 (SYN1) in (**c**) the hippocampus and (**d**) the PFC ($n=4$ per group; $^{**}P<0.001$; $^{\#}P<0.01$). mRNA expressions of *Ncam1* and *Syn1* were measured by quantitative PCR (qPCR). Data represented as mean ± s.e.m. Asterisk represents the effect of methylazoxymethanol (MAM) in every experimental group. Double asterisk represents the comparison between control and chronic mild stress (CMS) groups. Cardinal represents the effect of antidepressant treatment.

imipramine ($P=0.006$), CP 156,526 ($P=0.001$) or SSR 149415 ($P=0.001$), but not fluoxetine, reversed the CMS-induced increases in the percentage of thin-type spines ($F_{1,\ 20}=32.57$, $P<0.001$). Treatment with MAM, alone or in combination with CMS and/or antidepressants did not result in any significant changes in dendritic morphometry in either the hippocampus or the PFC. No significant changes in spine morphology were observed in basal dendrites of pyramidal neurons from the CA3 region of the hippocampus and the PFC (data not shown).

Gene expression studies

The mRNA expression of *Ncam1* and *Syn1* was measured by qPCR. The expression of *Ncam1* was significantly reduced in the hippocampus of CMS rats ($F_{1,\ 12}=73.18$, $P<0.001$). These changes were reversed by fluoxetine, imipramine, CP 156,526 and SSR 149415 ($P<0.01$ in all cases; Figure 4a). Furthermore, rats exposed to CMS revealed significantly reduced levels of *Ncam1* expression in the PFC ($F_{1,\ 12}=28.73$, $P<0.001$) that were reversed by all antidepressants ($P<0.01$ in all cases; Figure 4b). Co-administration of MAM significantly decreased *Ncam1* mRNA levels in the hippocampus of all experimental groups ($F_{1,\ 46}=8.617$, $P=0.005$); MAM did not influence *Ncam1* expression in the PFC. Exposure to CMS, with/without concomitant treatment with MAM and/or antidepressants, did not alter *Syn1* mRNA expression in the hippocampus (Figure 4c). However, *Syn1* expression was significantly reduced in the PFC of rats exposed to CMS ($F_{1,\ 12}=23.27$, $P<0.001$) and reversed by administration of fluoxetine, imipramine, and SSR 149415, ($P<0.01$ in all cases) but not of CP 156,526 (Figure 4d). With the exception of the group that had been exposed to CMS, treatment with MAM led to a significant decrease in *Syn1* mRNA expression in the PFC of all experimental groups ($F_{1,\ 46}=12.809$, $P=0.001$).

Discussion

The behavioral data obtained confirmed the validity of CMS as an animal model of depression insofar that animals exposed to stress exhibited clear signs of anhedonia (reduced sucrose preference) and learned helplessness (increased immobility in the FST). Furthermore, the administration of four antidepressant drugs with different mechanisms of action significantly attenuated these depressive-like behaviors after 1 week (imipramine, CP 156,526 and SSR 149415) or 2 weeks of treatment (fluoxetine). The analysis of cell proliferation and differentiation in the hippocampus confirmed previous reports of the antineurogenic effects of CMS on the one hand,[2,9] and the proneurogenic effects of the antidepressant compounds on the other.[8–10] Briefly, the hippocampi of CMS rats revealed significantly reduced overall numbers of recently-mitotic (Ki67-positive and BrdU-positive) cells, a proportion of which were recently-

generated neuroblasts (Ki67-positive/DCX-positive) and young neurons (BrdU-positive/NeuN-positive); CMS did not influence the number of newly born astroglial cells (BrdU-positive/GFAP-positive). Antidepressant treatment not only reversed the antiproliferative effects of CMS, but stimulated neurogenesis to levels above those found in controls. These behavioral actions of antidepressants were observed in animals displaying signs of depressive-like behavior, contrasting with some previous reports that antidepressants can elicit behavioral effects under basal conditions.[8,10,16] Although temporal coincidence in the occurrence of impaired neurogenesis and depressive-like symptoms and their reversal by antidepressant treatment is evident, these data do not demonstrate a direct cause effect between neurogenesis and the alleviation of signs of depressive-like behavior, and recently it was shown that this action of antidepressants is mediated indirectly by neurotrophins.[35] To examine whether antidepressant-induced neurogenesis may simply be an epiphenomenon, we tested the therapeutic efficacy of the various antidepressants in subsets of rats that were concomitantly treated with MAM. This cytostatic agent was chosen because X-ray irradiation[17] is likely to induce inflammation and requires a period of recovery before antidepressants can be administered. Pilot studies showed that MAM at a dose of $7\,mg\,kg^{-1}$ per day reduces neurogenesis, without any undesired effects on general health. Importantly, the various antidepressants ameliorated CMS-induced behavioral signs of depression to the same extent in vehicle and MAM-treated animals. These observations indisputably demonstrate that the mood-improving actions of antidepressants occur independently of their ability to stimulate hippocampal neurogenesis.

Signs of anxiety are often present in both depressed patients and animal models of depression and many antidepressant drugs have anxiolytic properties.[36] Using the NSF paradigm, we found that all antidepressant drugs tested significantly reduced the hyperanxious state observed in CMS-exposed rats. Importantly, however, ongoing neurogenesis seems essential for the manifestation of this behavioral effect of antidepressants. This finding accords with results from Santarelli *et al.*[17] who, however, interpreted the results of this behavioral assay as a measure of antidepressive action. Neurogenesis in the hippocampus was previously shown to be essential for certain hippocampus-dependent functions, including trace memory formation[27] and spatial learning.[37] As the hippocampus sends major projections to the extended amygdala, it is plausible that new neurons, generated in response to antidepressant treatment, integrate into neuronal networks implicated in emotional behavior and thereby, modulate anxiety.

Structural changes within the hippocampus and the PFC are increasingly recognized as key to the pathophysiology of depression. Although total neuronal numbers in the hippocampus and in the PFC were not altered by any of the treatments, the CMS paradigm triggered significant volumetric changes in both regions. Accordingly, we next analyzed the potential contribution of synaptic plasticity and neuronal connectivity to the development of, and recovery from, depressive-like behavior. By cross-reference to the behavioral data concerning anhedonia and learned helplessness, it emerges that the expression of depression-like behavior in CMS rats tracks dendritic atrophy and loss of synaptic contacts. Interestingly, even though spine densities were not altered in any of the experimental groups, we observed a shift in the ratio between mushroom and thin spine types, suggesting the possibility of a change in spine turnover, that is, a subtle but significant, reflection of altered synaptic function. Another important finding was that antidepressant administration to CMS-treated rats concomitantly reversed signs of depression-like behavior and restored synaptic connections to a level found in control animals; these adjustments occurred independently of the neurogenic status.

Besides the structural changes within the hippocampus and the PFC, the present results support the view[22,24] that some of the behavioral disturbances in depressed subjects may result from an interruption of balanced communication between these, and other brain regions. As compared to controls and antidepressant-treated animals, CMS rats showed marked atrophy of the apical dendrites of neurons in the hippocampus (dentate gyrus and CA3 areas) and the PFC (layers II/III, upon which the hippocampal inputs impinge). The topographic distribution of the dendritic changes in the PFC observed after CMS and following antidepressants indicate that interruption of hippocampus-PFC connectivity may underlie the manifestation of depressive-like behavior.[22,38] Interestingly, the dendritic atrophy and regrowth observed in this study are consistent with reports in depressed patients of reduced hippocampal and PFC volumes[21,23] as well as their pharmacological reversibility[39] (but see reference[40]). In this context, our results reinforce the notion that antidepressants may facilitate rewiring of neural circuits, as demonstrated recently in amblyopic animals.[41]

Restoration of synaptic plasticity, reflected in increased levels of expression of NCAM1,[42,43] is thought to be central to the therapeutic efficacy antidepressant drugs.[1–3] Consistent with this view, all antidepressant drugs tested in this study reinstated hippocampal *Ncam1* expression in CMS-treated animals to levels found in control animals. Our observation of reduced levels of *Ncam1* in the hippocampus of MAM-treated animals concurs with the fact that newly born neurons transiently express *Ncam1*.[44] On the other hand, as postnatal neurogenesis does not occur in the PFC, the increased levels of cell adhesion molecules found in the PFC of antidepressant-treated rats (with or without concomitant dosing with MAM) supports previous suggestions that synaptic remodeling and plasticity may underlie the action of antidepressant drugs.[45] The observation

that the expression levels of the gene encoding for the presynaptic protein SYN1 were only altered in the PFC, together with our three-dimensional analysis of dendritic morphology and the results from a previous study,[24] suggest that remodeling of synapses located on the apical dendrites of pyramidal cells of PFC layers II/III may be important in the onset and amelioration of depressive-like behavior.

In summary, the present results demonstrate that antidepressants, irrespective of their mechanisms of action, trigger neuronal remodeling and synaptic plasticity. Moreover, we show that antidepressants promote hippocampal neurogenesis but that this is not a critical event for their mood-rectifying actions.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)